Thermoelectrically Cooled Temperature-Gradient Apparatus for Comparative Cell and Virus Temperature Studies

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Establishment of a near-linear temperature gradient in an incubator has been accomplished by the application of heat to one terminus of a conducting body, normally a metal bar, and the removal of heat from the other terminus of the conducting body. Such incubators have been complex and unwieldy because of the need for mechanical refrigeration. We have described a simplified temperature gradient incubator which uses thermoelectric module cooling coupled with electric heating. Along the gradient, 20 stations in two parallel rows of 10, each accommodating a 30-ml plastic cell culture flask, were continually monitored by an electronic thermometer, and the temperatures were recorded. By manipulation of two simple potentiometer controls, any temperature gradient between 0 and 50 C could be obtained. Minor deviations which occurred between theoretically perfect and obtained temperature gradients were reproducible and readily measured. The gradient incubator was particularly applicable to (i) simultaneously studying a given biological activity over the entire temperature range supporting the growth of a given cell, virus, or microorganism, or (ii) precisely defining the upper or lower temperature limits of a biological system by 10-point determinations. Preliminary experiments have demonstrated the usefulness of the apparatus in characterizing the temperature limits for growth in vitro of cells of reptilian cell lines. The gradient incubator was also successfully utilized for the characterization of the effect of temperature on the efficiency of plating of amphibian viruses and possible temperature variants of those viruses.

Comparative studies of the effect of different incubation temperatures on the efficiency of virus replication have revealed consistent differences in response, which have served as laboratory "markers" useful in distinguishing different strains of many mammalian viruses. Particular interest has attended frequent observations [reviewed by Brown (2) and Lwoff (16)] that a capacity for growth at high temperatures may often be correlated with a high degree of virus virulence expressed in vivo. The study of the effect of temperature on cell and virus growth has become of increasing theoretical interest with the recent establishment of many cell lines and the isolation of numerous viruses from poikilothermic vertebrates. The effect of temperature on the replication of viruses in host cells with optima

differing from that of the virus is of particular interest (7).

Interest centers on the optimal and limiting temperatures for the growth of cells and viruses. These data can be most desirably determined when all measurements are internal to a single experiment performed along a continuous temperature gradient. Several types of gradient incubators have been previously described, all based on an aluminum or stainless-steel block heated at one extremity (1, 2, 4, 10–14). Near-linear gradients were obtained only when the opposite extremity of the gradient was cooled by refrigerant pumped through the apparatus or by immersion in a cold water bath (1, 4, 10–13).

This report describes an entirely electronic gradient incubator with electric heating and thermoelectric cooling. The gradient obtained is determined by two simple controls. No water baths, compressors, or pumps are required.

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Results of preliminary experiments on the application of this instrument to virus and cell culture problems are also described.

MATERIALS AND METHODS

Reversible 10-station gradient incubator. An external view of the total gradient apparatus is shown in Fig. 1. The temperature gradient bar mechanism is illustrated in Fig. 2. The gradient incubator (Fig. 1, A) consists of a 0.5-inch ($\sim$1.3 cm) rolled aluminum bar [8 by 24 inches ($\sim$20.3 by 61 cm)] housed in a horizontal metal cabinet and insulated on all sides with polyethylene foam. The top has two parallel rows, each with 10 openings at 2.5-inch (6.4-cm) intervals along the length of the bar. Each opening accepts one plastic 30-ml tissue culture flask (Falcon Plastics Co.) placed directly on the aluminum bar. Each opening has an insulated lid.

To the underside of each end of the aluminum bar (Fig. 2, point 7) are attached six thermoelectric modules operated by DC power [Cambridge Thermionic Corp., model 800-3951-01 (Fig. 2, point 3)]. Four electrical cartridge heaters [Vulcan NC 252 series; 50 w, 120 v (Fig. 2, point 4)], two on each side, are placed in holes drilled in the aluminum bar directly above the thermoelectric cooling modules. A finned heat exchanger (Fig. 2, point 2) is used to dissipate the heat which is generated by the thermoelectric modules and cartridge heaters. A blower (Fig. 2, point 1) is used to remove the heat dissipated into the heat exchanger. Wooden legs (Fig. 2, point 6) are used to support the bar and temperature source assembly to minimize heat loss by conduction from the apparatus.

At each end of the bar next to one heating cartridge is a thermistor (Fig. 2, point 5) used to sense the temperature of the bar. As the device temperature increases or decreases, the thermistor resistance de-
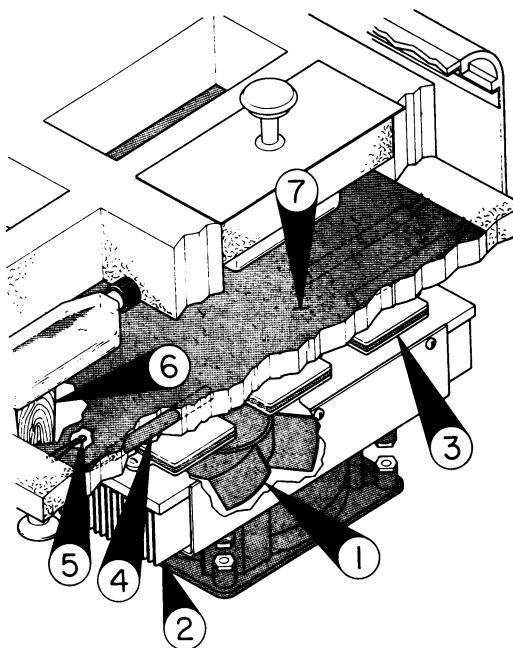

FIG. 2. *Detail of gradient incubator terminal assembly. Each end of aluminum bar (7) contains cartridge heaters (4) and thermoelectric cooling modules (3), accompanied by a finned heat exchanger (2) cooled by a fan (1). Temperature is monitored by adjacent thermistor (5). Apparatus is supported by low conductivity wooden legs (6).*

creases or increases, appropriately signaling the temperature controller (Fig. 1, B).

To avoid abrupt changes as the temperature is regulated, a temperature controller performing on the principle known as proportional action is employed. The controlling action depends on the *difference* in value between the controlled variable (temperature) and the dialed setting. The greater the difference, the higher the proportional action taken by the controller to drive the variable to the desired value. Control action proceeds continuously to adjust the voltage supplied to the cartridge heaters to balance heat losses.

Temperature read-out is provided by 10 thermistor probes (Yellow Springs Instrument Co., no. 402) embedded in grooves in the upper surface of the aluminum bar in the center of each cell culture flask position of the rear row. The probes are connected by means of coaxial connectors and cables to the first 10 positions of an 11-jack electronic thermometer (YSI Co., model 47 Telethermometer; Fig. 1, C). The 11th position can be used to monitor a thermistor probe cemented to the inner surface of a cell culture flask containing fluid or solid overlay cell culture medium. This flask inserted in the apparatus provides data on actual temperatures obtained in cell cultures under experimental conditions.

The Telethermometer automatically scans the 11 probe positions at a selected speed. The Telethermometer output can be recorded on a suitable strip
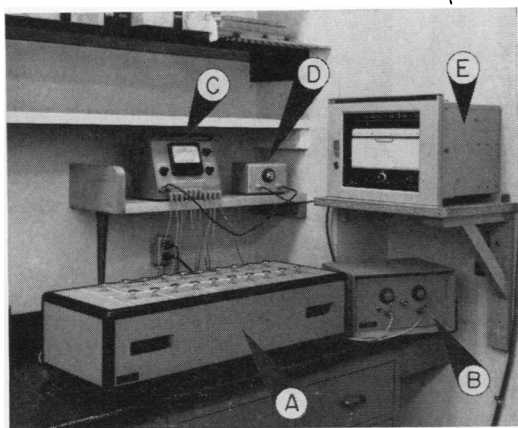

FIG. 1. *Temperature gradient apparatus. A, Temperature gradient incubator; B, temperature controller; C, electronic 11-jack scanning thermometer; D, voltage attenuator for adjustment of thermometer signal for recorder; and E, strip chart recorder.*

chart recorder, such as the Esterline Angus model E 1101 E (Fig. 1, E), by inserting a Cambion attenuator (Fig. 1, D) between the Teletthermometer and the recorder.

Cell culture. Cells of poikilothermic vertebrate cell lines were propagated in Eagles basal medium containing 10% fetal calf serum by methods previously described (8). *Terrapene* heart (TH-1) sublines A and B$_2$ (9) were grown at 23 C, whereas subline TH-1W was grown at 30 C. *Iguana* heart (IgH-2) cells (H. F. Clark, M. M. Cohen, and D. T. Karzon, Proc. Soc. Ex. Biol. Med., *in press*) were grown at 36 C. Chick embryo fibroblast (CEF) cell cultures were prepared by methods previously described (3) and grown in Earles salt solution containing 0.075% NaHCO$_3$, 0.25% lactalbumin hydrolysate, and 4.0% calf serum.

In gradient experiments, replicate flasks were seeded with 2.0 × 10^5 to 7.5 × 10^5 cells in 5.0 ml of growth medium and placed immediately in the gradient incubator. Two additional flasks were incubated at 30 C for 2 hr [at conditions previously determined to allow near 100% attachment (9)] and counted for a baseline cell number. Cultures in the gradient incubator were examined daily. When the fastest growing culture became nearly confluent (3 to 4 days), cells in all flasks were counted by a modification of the technique of Sanford et al. (17). Each flask was washed twice with phosphate-buffered saline (Dulbecco) and drained dry. Five milliliters of crystal violet (0.01% in 6.0% citric acid) was added to each flask; the flasks were then agitated for 5 min at 60 cycles per min on a mechanical shaker. Whole nuclei were then counted in a hemacytometer. The increase in cell numbers expressed as log$_2$ was divided by the number of days of growth and taken as the number of cell doublings per day.

Virus. The amphibian cytoplasmic virus LT1 was isolated from a newt (*Triturus viridescens*) inoculated with kidney tumor cells of *Rana pipiens* (6). Stocks were prepared in TH-1B$_2$ cells incubated at 23 C or on the chorioallantoic membranes (CAM) of embryonated hens' eggs incubated at 30 C. Stocks of the amphibian cytoplasmic virus T6, isolated from a grossly normal newt (H. F. Clark, C. Gray, F. Fabian, R. F. Zeigel, and D. T. Karzon, Recent Results in Cancer Research, Springer-Verlag, *in press*), were prepared in TH-1B$_2$ cells at 23 C.

Virus plaque assays were performed by the method of Lehane et al. (15). Starch overlay was added after adsorption of 100 to 200 plaque-forming units (PFU) of virus per flask for 1 hr at 23 C. A staining overlay containing neutral red was added on the 6th day after inoculation, and plaques were counted on the 8th day. Efficiency of plaquing (EOP) at each temperature tested was expressed as the percentage of the maximal number of plaques obtained at any of the 10 temperatures tested.

RESULTS

Gradient apparatus performance. In practice, an experimental temperature gradient is established by fixing the appropriate terminal temperatures of the bar with the two potentiometer

controls, according to predetermined calibration curves. Fine adjustments are then performed according to the exact temperatures obtained from the teletthermometer.

Because insulation of the gradient incubator is not absolute, obtained temperature gradients vary slightly from the theoretically perfect value, with the variation increasing with the difference from ambient. Practically, this does not present a problem, inasmuch as real incubator temperatures are recorded and these may be readily checked against actual flask temperatures by using the thermistor-containing flask. Experimental results are always expressed in terms of the actual flask temperatures determined at each position of a given gradient.

Near-straight line relationships are obtained when gradients spanning ambient temperature (Fig. 3) or involving cooling at each terminus are employed. When gradients requiring heating at each end of the incubator are employed (Fig. 4), peripheral heat loss causes the position adjacent to the lower temperature terminus (no. 2 rather than no. 1) to give the lowest temperature. Nevertheless, a linear gradient is then obtained from positions 2 to 9 or 10. Since results are plotted according to temperature rather than position, all data obtained are still usable.

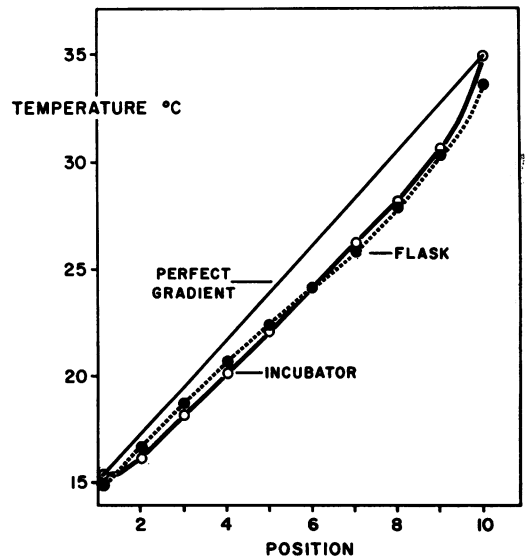By using a given set of terminal temperatures, the temperatures obtained at intermediate posi-

FIG. 3. *Typical experimental temperature gradient with temperature range spanning ambient. Incubator temperatures are monitored by thermistors embedded in aluminum bar of gradient incubator, and flask temperatures are measured with thermistor within cell culture flask.*
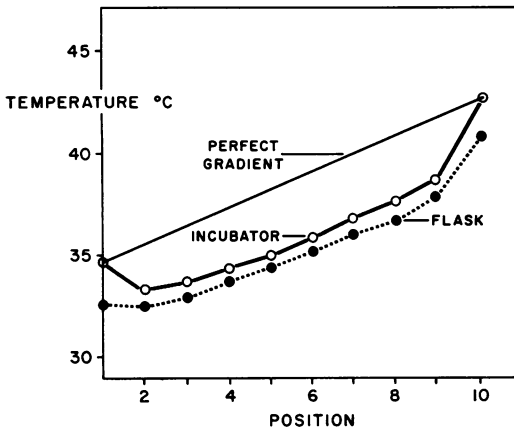
FIG. 4. *Representative temperature gradient obtained between two temperatures above ambient.*

tions on the gradient incubator are reproducible within the accuracy of reading the telethermometer (approximately ± 0.1 C). The variation between given front row and rear row positions is ≤ 0.2 C. The temperature drift of the instrument does not normally exceed ± 0.2 C in a 24-hr period, provided that ambient temperatures do not vary.

Cell culture studies. In previous studies (9), the 23 C TH-1 cell subline A was shown to be capable of growth for a few generations only at 30 C and incapable of growth at 36 C. Another 23 C TH-1 cell subline, B_2, was capable of indefinite growth at 30 C and limited growth at 36 C. A 30 C subline of B_2, which was transferred to the warmer temperature at the 41st passage, was designated W. The effect of incubation temperatures attained on a 10 to 45 C gradient on the growth of these three cell sublines (A, 73rd passage; B_2, 102nd passage; W, 157th passage) was determined in the gradient incubator.

The results (Fig. 5) indicated that, although cells of all sublines grew at temperatures ranging from 23.0 to 33.7 C, multiplication of cell subline A was depressed at 33.7 C, whereas B_2 and W cells grew at maximal rates. None of the cells grew at 37.2 C. W cells, which showed the highest growth rate at 33.7 C, were also the only cells to multiply demonstrably at the low temperature of 20 C.

A 36 to 46 C gradient was employed to provide a detailed analysis of maximal temperature tolerance of *Terrapene* B_2 cells and the cells of *Iguana* cell line IgH-2 established at 36 C (Fig. 6). Growth of the *Terrapene* cells was demonstrated at temperatures up to 35.0 C but not at 35.9 C. The *Iguana* cells multiplied at temperatures as high as 38.0 C, but not at 39.1 C.

Virus studies. In gradient experiments reported elsewhere (Clark, Gray, Fabian, Zeigel, and Karzon, Springer-Verlag, *in press*), the effect of temperature on the EOP of several amphibian cytoplasmic virus strains of diverse host origin was determined. The different virus strains could not be distinguished on the basis of their temperature optima for plaquing, or the upper or lower limits of temperature supporting plaque formation. Those experiments employed plaquing in TH-1 cells of virus propagated in TH-1 cells at 23 C.

Further gradient experiments were performed to investigate the effect of host cell on the EOP at different temperatures. EOP curves obtained with amphibian virus LT1 before and after two passages at 30 C in the chick embryo chorioallantoic membrane were determined in TH-1 and CEF cell cultures (Fig. 7).

The EOP of virus of each stock of LT1 in CEF cells was markedly lower than that in TH-1
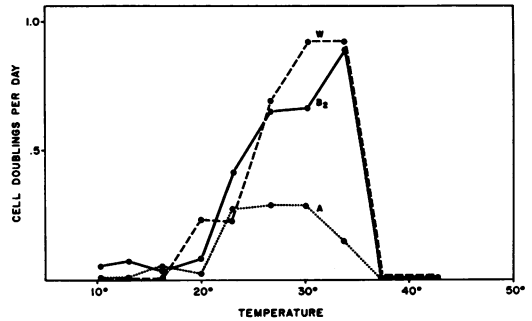

FIG. 5. *Gradient determination of the effect of temperature on the growth rate of cells of Terrapene cell line TH-1, sublines A, B_2, and W.*
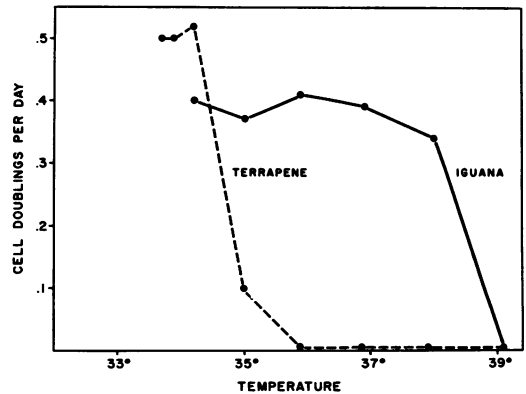

FIG. 6. *Gradient determination of the temperature limits supporting growth of cells of Terrapene (temperature turtle) cell line TH-1 and Iguana (tropical lizard) cell line IgH-2.*
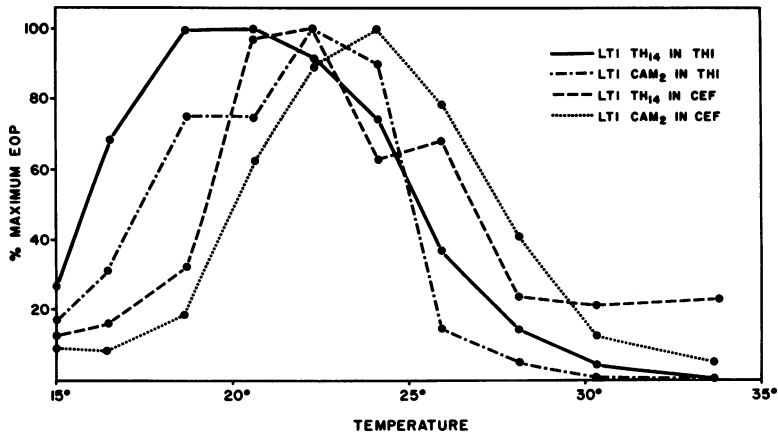
FIG. 7. *Gradient determination of temperature effect on efficiency of plaquing (EOP) of amphibian virus LT1 in cells of Terrapene cell line TH-1 and in chick embryo fibroblasts (CEF). LT1 TH$_{14}$ virus was propagated exclusively in TH-1 cells at 23 C; LT1 CAM$_2$ virus was also passaged twice in chick embryo chorioallantoic membrane at 30 C.*
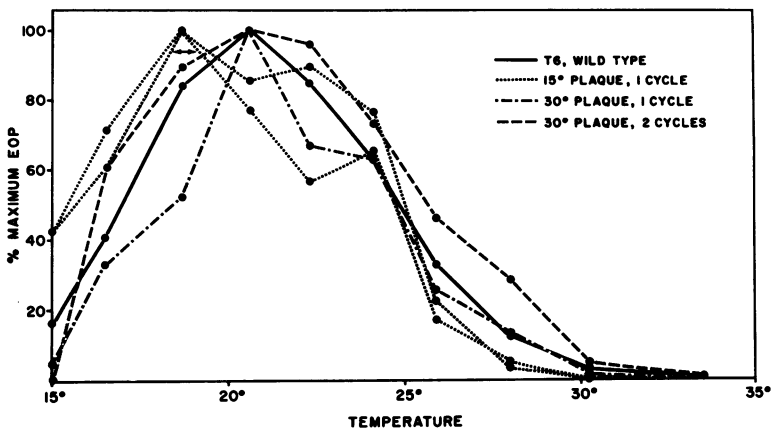

FIG. 8. *Gradient determination of temperature effect on the efficiency of plaquing (EOP) of clones of amphibian virus T6 derived from plaques appearing at the upper and lower temperature limits (15 and 30 C) supporting plaque formation by the wild type. Determinations for two 15 C plaques (cycle 1) are illustrated.*

cells at temperatures below 20 C. At temperatures above 25 C, the EOP in CEF cells was consistently enhanced. Plaques were obtained in CEF cells at 33.6 C, a temperature nonpermissive for plaque formation in TH-1 cells. Passage of LT1 virus twice in CAM at 30 C led to a slightly depressed EOP at low temperatures, but the EOP was not consistently elevated at the warmer end of the gradient.

The gradient incubator was employed in further preliminary experiments designed to determine whether plaques caused by amphibian cytoplasmic viruses at upper and lower temperature limits might represent true temperature mutants. An uncloned "wild" stock of newt isolate T6 (maintained by passage of undiluted

frozen and thawed infected cell suspension in TH-1 cells at 23 C) was plaqued in TH-7 cells on a 15 to 35 C gradient. Several plaques appearing at 15 C and at the highest permissive temperature (30.3 C) were harvested, used to prepare new stocks in TH-1 cells incubated at 15 and 30 C, respectively, and replaqued on the temperature gradient. In a single experiment, a second cycle of selection and retesting of 30 C plaques was performed.

Representative results are illustrated in Fig. 8. EOP curves obtained with the progeny of 15 and 30 C plaques remained basically similar to those obtained with the wild virus. Virus derived from 30 C plaques exhibited the same optimal temperature as the wild virus and the same maximal per-

missive temperature. The 30 C derived virus exhibited depressed EOP at 15.0, but not at 16.5 C. Virus of 15.0 C plaque origin consistently exhibited slightly elevated EOP at temperatures below 20 C and depressed EOP above 25 C. The results suggest that temperature reproductive capacity (RCT; reference 5) determinations performed at 30 and 15 C might serve to distinguish "hot" and "cold" variants of amphibian cytoplasmic viruses.

It is not clear whether the minor extent of the variation from normal temperature response observed in the progeny of "hot" and "cold" plaque viruses truly reflects the genotype of the selected plaque variants, or whether it results from a high rate of reversion to "wild" virus type, possibly occurring during plaque virus outgrowth before testing.

DISCUSSION

An apparatus has been described which allows simultaneous incubation of duplicate sets of cell culture flasks at 10 different temperatures along any gradient within the limits of 0 to 50 C. Since the apparatus is electronically cooled and heated, it requires no mechanical refrigeration. Operation requires only connection to a 110-v power source and the adjustment of the terminal incubation temperatures with two simple dial controls. Because of the simplicity of its installation and operation, this apparatus represents a considerable advance in temperature gradient incubator design.

It has been demonstrated that near-linear temperature gradients are obtained in the incubator when ranges entirely above, entirely below, or including the ambient are employed. Temperatures obtained at each of the 10 stations along the gradient have been shown to be reproducible for any given set of terminal temperatures tested. Real temperatures obtained in cell culture flasks may vary slightly from those recorded by thermistors in the apparatus because of the poor thermal conductivity of the plastic containers and the influence of ambient temperatures. However, these discrepancies are constant for each point on a given gradient and are readily measured by means of a thermistor placed inside a cell culture flask.

Most studies of the effect of temperature on cells and viruses have been limited to a few arbitrarily selected temperatures by the practical limitation on the number of incubators available. Many studies of hot or cold mutants have employed two temperatures only. The gradient incubator can be utilized to simultaneously provide data at 10 points encompassing the entire temperature range tolerated by a given cell or virus

population, or to define precisely the behavior of a system at 10 points, including its upper or lower temperature limit only.

Thus, the gradient data presented in a single experiment revealed that a turtle cell line, previously shown to have acquired tolerance for high incubation temperatures (9), (i) had a temperature optimum higher than the parental cell line, (ii) grew more rapidly than the parental cell line at all temperatures, and (iii) also grew at low temperatures nonpermissive for the parental cell line. In a limit-determining type of experiment, it was simultaneously determined that cell line TH-1 B_2 derived from the temperature-climate box turtle had a growth limit between 35.0 and 35.9 C, whereas cell line IgH-2 derived from the tropical green iguana had a limit between 38.0 and 39.1 C.

Data have also been presented, demonstrating the usefulness of the gradient incubator in determining the effect of temperature on the EOP of viruses. The apparatus is equally applicable to studying the effect of temperature on virus growth rates and yield (Clark, *unpublished data*).

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